PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.: 10/782,570 Confirmation No.: 5780
Applicant(s): Carozzi et al.
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Art Unit: 1638
Examiner: Anne R. Kubelik
Title: AXMI-007, A DELTA-ENDOTOXIN GENE AND METHODS FOR ITS USE

Docket No.: APA016US01
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Commissioner for Patents
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APPEAL BRIEF UNDER 37 CFR §41.37

This Appeal Brief is filed pursuant to the Notice of Appeal mailed on August 16, 2011, in response to the Final Office Action mailed May 18, 2011.

1. ***Real Party in Interest.***

The real party in interest in this appeal is Athenix Corp., the assignee of the above-referenced patent application.

2. ***Related Appeals and Interferences.***

Application Serial Nos. 10/782,096, 10/782,141, and 10/783,417 (each filed February 19, 2004) are being appealed concurrently herewith. While each of these applications is independent, the issues on appeal are identical.

3. ***Status of the Claims***.

Claims 1-11, 19, 22, and 23 are the subject of this appeal. Claims 1-11, 19, 22, 23, and 30 have been rejected. Claims 12-18, 20, 21, and 24-29 have been canceled.

4. ***Status of Amendments.***

No new amendments have been made to the claims.

5. ***Summary of Claimed Subject matter.***

Independent claims 1, 22 and 23

Independent claim 1 is drawn to an isolated or recombinant nucleic acid comprising a nucleotide sequence encoding an amino acid sequence having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:2 or 4, wherein said polypeptide has pesticidal activity against lygus pests. Support for claim 1 can be found, at least, on page 17, lines 17-20; page 30, lines 16-20, and Experimental Example 10.

Independent claim 22 is drawn to a plant having stably incorporated into its genome a DNA construct operably linked to a promoter that drives expression of a coding sequence in a plant cell, wherein the DNA construct comprises a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2 or 4, wherein said polypeptide has pesticidal activity against lygus pests. Support for claim 22 can be found, at least, on page 3, lines 2-26; page 17, lines 17-20; page 20, lines 3-7; page 30, lines 16-20, and Experimental Example 10.

Independent claim 23 is drawn to a plant cell having stably incorporated into its genome a DNA construct operably linked to a promoter that drives expression of a coding sequence in a plant cell, wherein the DNA construct comprises a nucleotide sequence that encodes a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:2 or 4, wherein said polypeptide has pesticidal activity against lygus pests. Support for claim 22 can be found, at least, on page 3, lines 2-26; page 17, lines 17-20; page 20, lines 3-7; page 30, lines 16-20, and Experimental Example 10.

6. ***Grounds of Rejection to Be Reviewed on Appeal.***

(a) Issue 1 - Whether claims 1 and 4-7 are obvious over Ben-Dov *et al.* (1996) *Appl. Environ. Microbiol.* 62:3140-3145 in view of Liu et al. (U.S. Patent 6,156,308) and further in view of Carlton *et al.* (1985) Mol. Biol. Microb. Differ., Proc. Intl. Spore Conf., 9th, Meeting date 1984, pages 246-252, and further in view of deMaagd et al. (2001) *Trends Genet.* 17:193-199), and taken with the evidence of Applicants response to the Request for Information under 37 CFR 1.105.

(b) Issue 2 - Whether claims 2-3, 8-11, 19, 22, 23, and 30 are obvious over Ben-Dov *et al.* in view of Liu et al. and further in view of Carlton *et al.* and deMaagd et al., and further in view of Koziel *et al.* (U.S. Patent 5,625,136).

7. ***Argument***

(a) Issue 1 – Whether claims 1 and 4-7 are obvious over Ben-Dov *et al.* (1996) *Appl. Environ. Microbiol.* 62:3140-3145 in view of Liu et al. (U.S. Patent 6,156,308) and further in view of Carlton *et al.* (1985) Mol. Biol. Microb. Differ., Proc. Intl. Spore Conf., 9th, Meeting date 1984, pages 246-252, and further in view of deMaagd et al. (2001) *Trends Genet.* 17:193-199), and taken with the evidence of Applicants response to the Request for Information under 37 CFR 1.105.

The Examiner has rejected the claims under 35 U.S.C. § 103(a) and suggests that, at the time the invention was made, It would have been obvious to one of ordinary skill in the art to clone delta-endotoxin genes from strain HD536 described in Carlton et al. using the methods described in Ben-Dov et al. and Liu et al. The Examiner states that one of ordinary skill in the art would have sequenced the plasmid fragments and translated the resulting sequences to identify open reading frames, which would then have been compared to known Cry protein conserved sequence and structural domains, as taught by DeMaagd et al., to identify Cry

encoding reading frames. The Examiner also states that the use of probes made by the method of Liu et al. and designed from the protein sequences of toxins made by HD536 would have aided in cloning toxin genes, including those encoding SEQ ID NO:2 or 4, from that strain. For the following reasons, the Examiner's reasoning is not well founded and the rejection should be reversed.

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I. CLAIMS 1 AND 4-7 MEET THE REQUIREMENTS OF 35 U.S.C. § 103(a).

The rationale to support a conclusion that the claim would have been obvious is that all the claimed elements were known in the prior art and one skilled in the art could have combined the elements as claimed by known methods with no change in their respective functions, and the combination yielded nothing more than predictable results to one of ordinary skill in the art. *KSR Int'l Co. v. Tele-flex Inc.*, 550 U.S. 389 (2007); *Sakraida v. AG Pro, Inc.*, 425 U.S. 273, 282, 189 USPQ 449, 453 (1976); *Anderson's-Black Rock, Inc. v. Pavement Salvage Co.*, 396 U.S. 57, 62-63, 163 USPQ 673, 675 (1969); *Great Atlantic & P. Tea Co. v. Supermarket Equipment Corp.*, 340 U.S. 147, 152, 87 USPQ 303, 306 (1950). MPEP § 2143(A) (emphasis added).

A. One of ordinary skill in the art would have no motivation to combine Ben-Dov *et al.* together with Carlton *et al.* in view of Liu *et al.* and deMaagd *et al.*

At the outset, the Examiner impermissibly uses Applicant's Specification as a basis for the rejection. Specifically, the Examiner requested source information under 37 C.F.R. 1.105 and subsequently used this information as a basis for the obviousness rejection under 35 U.S.C. § 103(a).[1] However, outside of Applicant's Specification, one of ordinary skill in the art would have no reason to select and isolate sequences from HD536 given the numerous possibilities of well-known strains exhibiting insecticidal activity. Moreover, the only motivation for even considering sequences isolated from HD536 comes from the instant disclosure and not the cited references. Without a

[1] Applicants respectfully note that the response to the Request for Information under 37 CFR 1.105 was submitted to the USPTO and was labeled as proprietary material with the label "DO NOT SCAN." Applicants respectfully disagree with the Examiner's inclusion of this material in the Final Office Action dated January 12, 2010.

specific teaching or motivation, one of ordinary skill in the art would not even look to Carlton *et al.* to choose a strain, let alone select HD536 from the numerous possibilities included in Carlton *et al.*

i. *Ben-Dov et al. do not teach or suggest all of the claimed elements.*

The Examiner acknowledges that Ben-Dov *et al.* alone is deficient to render the claims obvious and states that "Ben-Dov et al. do not teach a nucleic acid encoding SEQ ID NO: 2, 4 or 6." Office Action dated December 6, 2010, at page 3. At best, Ben-Dov *et al.* teach cloning of large restriction fragments from *Bacillus thuringiensis* subsp. *israelensis* and identification of known toxins using Southern hybridization and probes specific for the known toxins. Ben-Dov *et al.* does not teach or suggest a nucleic acid molecule encoding polypeptide with activity against lygus pests, let alone the claimed sequences. Rather, Ben-Dov *et al.* is concerned with characterizing a single 125-kilobase plasmid containing genes that encode delta-endotoxins with activity against mosquito larvae. Ben-Dov *et al.* at page 3140.

ii. *Carlton et al. fail to make up for the deficiencies of Ben-Dov et al.*

Carlton *et al.* evaluates a large number of *Bacillus thuringiensis* strains for the presence of extrachromosomal DNA by agarose gel electrophoresis. Carlton *et al.* at page 251 and Figure 1. In doing so, Carlton *et al.* sets forth a laundry list of *Bacillus thuringiensis* plasmids, including HD536, together with the estimated size of the plasmids in Table 1. Carlton *et al.* at Table 1. In rejecting the claims, the Examiner cites to Table 1 and states that "Carlton et al. teach that strain HD536 has a 68 MDa plasmid implicated in toxin production." Final Office Action at page 5. The Examiner further asserts that "it would have been obvious to one of ordinary skill in the art to modify the method of cloning delta-endotoxin genes from *B. thuringiensis* plasmids as taught by Ben-Dov et al, to clone delta-endotoxin genes from strain HD536 described in Carlton et al." *Id.* The Examiner also states that "[o]ne of ordinary skill in the art would have been motivated to do so because an increased repertoire of delta-endotoxins would be desirable for increasing toxicity spectra and for overcoming pest resistance to existing endotoxins." *Id.* Applicants respectfully disagree.

iii. *Liu et al. fail to make up for the deficiencies of Ben-Dov et al.*

Liu et al. used PCR analysis using probes specific to a series of known cry genes to profile strains. Nonetheless, no genes were isolated or identified by Liu et al. using this method. Instead, Liu et al. tested protein isolates and partially sequenced two protein bands fractionated from these isolates. Thus, even when provided the HD536 strain taught by Carlton et al., one of skill in the art would be unlikely to utilize the methods taught by Liu et al. to identify *every single* gene present in HD536, let alone the genes of the instant invention as suggested by the Examiner. Thus, Liu et al. provide no direction or guidance for identifying the specific delta-endotoxin gene claimed in the instant invention.

iv. *There would have been no reasonable expectation of success in arriving at the claimed invention by combining the teachings of Ben-Dov et al. with Liu et al. and Carlton et al.*

One of ordinary skill in the art would have no motivation to combine the teachings of Ben Dov *et al.* with Carlton *et al.* and Liu *et al.* in a manner that renders the claims obvious. For one, Ben Dov *et al.* is limited to evaluating a specific 125-kilobase plasmid containing known genes that encode delta-endotoxins with activity against mosquito larvae. Ben-Dov *et al.* at page 3140. To achieve this, Ben-Dov *et al.* utilizes probes capable of specifically identifying genes of interest that encode delta-endotoxins with specific activity against mosquito larvae. Ben-Dov *et al.* provides no additional motivation to apply this methodology to toxins other than those that are active against mosquito larvae. Ben-Dov *et al.* also acknowledges that "[a]mbiguous results were obtained with several additional probes (data not shown)…", thus indicating further uncertainty regarding the methodology and applicability of the teachings of Ben-Dov *et al.* Given the above, one or ordinary skill in the art would have no motivation to modify and apply the methodology of Ben-Dov *et al.* to other plasmids, such as those set forth in Carlton *et al.*, with a reasonable expectation of success.

Further, Ben-Dov also used hybridization methods based on the sequence of known genes, and even then was unsuccessful in identifying anything other than very highly similar genes. This

method requires the presence of sequences with a high degree of homology to the existing genes, at least in the region corresponding to the probe sequences. The gene claimed in the instant invention has low homology to the genes known at the time of filing. Thus, there would have been no reasonable expectation of success in identifying the axmi gene of the invention using the methods taught by Ben-Dov et al.

Liu et al. also used probes specific for the known cryIA(a), cryIA(b), cryIA (c), cryID, cryIIIA, cryIIIB, cryIIIC gene, and cryIIID genes (Example 3 of Liu et al.) to profile two *Bacillus thuringiensis* strains. Liu et al. also sequenced protein fragments from the crystal protein fraction of the strains (Example 8), and used the sequence information to design degenerate oligonucleotides to identify the coding sequence for the protein (Example 9). Thus, using these methods, one of skill in the art can only identify either already known genes, or genes that are highly expressed from these Bt strains, and further elucidation of toxin genes may be prevented by the "masking" of lower-expressed genes by the more highly expressed toxins in a particular strain. Thus, Liu et al. provide no direction or guidance for identifying the specific delta-endotoxin gene claimed in the instant invention. While methods for isolating toxin genes from strains having pesticidal activity were known in the art at the time of filing of the instant application, Applicants maintain that there would have been no reasonable expectation of success in identifying the specific genes claimed in the instant invention.

B. One of ordinary skill in the art would have no motivation to use the specific probes of Ben-Dov *et al.* to isolate SEQ ID NO:1-4 from HD536

In rejecting the claims, the Examiner cites to Carlton *et al.* and states that "[i]t is obvious to use the 68 mDa plasmid from HD536 because HD536 was known in the art as having a toxin-encoding plasmid" and "...one of skill would necessarily isolate a nucleic acid encoding SEQ ID NO:2 or 4." Office Action at page 4. Applicants respectfully disagree. One of ordinary skill in the art would have no motivation to use the methodology and specific probes of Ben-Dov *et al.* in an attempt to isolate any one of claimed SEQ ID NO:1-4 from HD536. For one, the claimed

sequences have a low sequence homology with other known toxins (<30%). As such, one of skill would not have used the cryIVA, cryIVB, cryIVC, cryIVD, and cytA, probes taught in Ben-Dov *et al.* to isolate any sequence from HD536, let alone the claimed sequences. In fact, only the cryIVA probe was able to detect any gene other than itself, and Ben-Dov *et al.* appears to attribute this cross-reactivity with the degree of sequence homology between the two genes. *See* Ben-Dov *et al.* at column 2, page 3143. Outside of pure conjecture, it is not clear how one of skill in the art would be able to use the hybridization method disclosed by Ben-Dov *et al.* to isolate the sequences of the invention.

C. One of ordinary skill in the art would have no motivation to try and isolate the claimed sequences from HD536 of Carlton *et al.*

A person of ordinary skill in the art would have no motivation to specifically select and isolate sequences from HD536, especially given the laundry list of plasmids described in Carlton *et al.* Additionally, HD536 is only mentioned once in Table 1 and Carlton *et al.* does not specifically indicate why it would be advantageous to isolate sequences from HD536, let alone the claimed sequences.

An "obvious to try" rationale may only support a conclusion that a claim would have been obvious where one skilled in the art is choosing from a finite number of identified, predictable solutions, with a reasonable expectation of success. *KSR Int'l Co. v. Tele-flex Inc.*, 550 U.S. 389 (2007). One of ordinary skill in the art would fail to recognize any reasonable expectation of success in obtaining any toxin genes from HD536 since *no insecticidal activity was demonstrated for this strain prior to the Applicant's disclosure*. Outside of providing a loose correlation between the ~68 MDa plasmid of HD536 and "toxin production," Carlton *et al.* fails to suggest that genes isolated from HD536 would have any insecticidal activity. Instead, Carlton *et al.* merely suggest that the 68 MDa plasmid present in strain HD536 may be responsible for crystal protein production. The presence of a crystal protein provides no evidence for the presence of a gene or encoded

protein having insecticidal activity against any pest, particularly any lepidopteran, coleopteran or heteropteran pests.

Further, the Federal Circuit has recently stated that a chemical structure (e.g., a polynucleotide or polypeptide) cannot be considered obvious unless the prior art suggests a lead compound *and modifications necessary to achieve the claimed molecule*. *Eisai Co. Ltd. V. Dr. Reddy's Laboratories, Ltd. and Teva Pharmaceuticals USA, Inc.,* No. 2007-1397, 2007-1398 (Fed. Cir. 2008).

In *Eisai*, Teva asserted that a combination of three prior art references rendered the claims of the '552 patent to rabeprazole and its salts obvious. The prior art references teach the compound lansoprazole, which differs from rabeprazole solely in the substituent at the 4th position of the pyridine ring. Teva argued that lansoprazole would have been selected by a person of ordinary skill in the art as a lead compound that could have been modified to produce rabeprazole. The Federal Circuit found that there existed no reason to substitute the fluorinated substituent of lansoprazole for the methoxypropoxy substituent of rabeprazole. In making this conclusion, the Federal Circuit stated that "KSR presupposes that the record up to the time of invention would give some reasons, available within the knowledge of one of skill in the art, to make particular modifications to achieve the claimed compound," (emphasis added) citing *Takeda* 492 F.3d at 1357. The Federal Circuit further states "obviousness based on structural similarity thus can be proved by identification of some motivation that would have led one of ordinary skill in the art to select and then modify a known compound (i.e., a lead compound) in a particular way to achieve the claimed compound" *Eisai*, emphasis added. As there is little similarity between the known cry toxins identified by Ben-Dov *et al.* and the claimed sequences, a person of ordinary skill in the art would not looked to the methodology of Ben-Dov *et al.* in an attempt to isolate the sequences of the instant invention from HD536.

D. AXMI-007 unexpectedly exhibits insecticidal activity against *Lygus lineolaris*

For at least the reasons set forth above, Applicants submit that the Examiner has failed to provide a *prima facie* case of obviousness. However, irrespective of this, secondary considerations of the advantageous properties of the claimed sequences, particularly the broad insecticidal activities of the recited sequences, provide additional support for the nonobviousness of the pending claims. See *In re Chupp*, 816 F.2d 643, 646, 2 USPQ2d 1437, 1439 (Fed. Cir. 1987) and *In re Papesch*, 315 F.2d 381, 137 USPQ 43 (CCPA 1963). Secondary consideration of superior results obtained with an invention provides objective indicia of nonobviousness. See, for example, *In re Mayne*, 104F.3d 1339, 1342, 41USPQ2d 1451, 1454 (Fed. Cir. 1997) and *In re Woodruff*, 919F.2d 1575, 1578, 16 USPQ2d 1934, 1936-37 (Fed. Cir. 1990). Such "secondary considerations" as those noted above further support the conclusion that the rejected claims are not obvious. For instance, Example 10 of the Specification provides evidence of the insecticidal efficacy of AXMI-007 (SEQ ID NO: 2) against *lygus lineolaris*. Specification, for example, at page 38, lines 8 – 24. As set forth in Example 10, samples containing the AXMI-007 protein in *Bacillus* yield a mortality rate of 50% against *lygus lineolaris* relative to a 0% mortality rate for the control. Specification at Table 2. This result is particularly unexpected given the relatively low amino acid identity of AXMI-007 as compared to the exemplary endotoxin classes described in Table 1 of the Specification (<30%).

(b) Issue 2 - Whether claims 2-3, 8-11, 19, 22, 23, and 30 are obvious over Ben-Dov *et al.* in view of Liu et al. and further in view of Carlton *et al.* and deMaagd et al., and further in view of Koziel *et al.* (U.S. Patent 5,625,136)

II. CLAIMS 2-3, 8-11, 19, 22, 23, AND 30 MEET THE REQUIREMENTS OF 35 U.S.C. § 103(a).

A. One of ordinary skill in the art would have no motivation to combine Ben-Dov *et al.* together with Carlton *et al.*

In rejecting Claims 2-3, 8-11, 19, 22-23, and 30 under 35 U.S.C. § 103(a), the Examiner acknowledges that "Ben-Dov et al in view of Liu et al. and further in view of Carlton et al and further in view of deMaagd et al. do not teach plants and seeds transformed with the nucleic acid." Office Action at page 5. However, the Examiner cites to Koziel *et al.* and asserts that it would have been obvious to one of ordinary skill in the art to transform the nucleic acid made obvious by Ben-Dov et al in view of Liu et al. and further in view of Carlton et al and further in view of deMaagd et al. into plants, including maize, as described in Koziel et al.

For at least the reasons set forth above, Applicants respectfully disagree with the Examiner's rejection of Claims 2-3, 8-11, 19, 22-23, and 30. Moreover, one of ordinary skill in the art would have no motivation to transform the nucleic acids taught by Ben-Dov *et al.* in view of Carlton *et al.* into plants or cells, let alone the specific claimed plants and cells. For one, Ben-Dov *et al.* has no association with plants and is solely concerned with isolating genes that encode toxins that are active against mosquito larvae. Ben-Dov *et al.* at page 3140. Accordingly, the emphasis in Ben-Dov *et al.* is centered around human infectious diseases as compared to plants or plant cells. *Id.* Outside of including HD536 in a list and describing a loose correlation between HD536 and toxin activity, Carlton *et al.* provides no motivation for including SEQ ID NO:1 or 3 in plants or cells. Koziel *et al.* fails to remedy the deficiencies of both Ben-Dov *et al.* and Carlton *et al.* and does not suggest transforming the claimed sequences into plants or cells. While Koziel et al. may provide a

general concept of transforming genes into plants, there is no suggestion that any of the cited references teaches or renders obvious transformation of plants or plant cells with the specific genes of the invention, since none of these references r ender obvious the genes themselves.

8. ***Claims Appendix.***

An appendix containing a copy of the claims involved in the appeal.

9. ***Evidence Appendix.***

An Appendix containing copies of the evidence submitted as follows:

Evidence Appendix A: Ben-Dov *et al.*, 1996

Evidence Appendix B: Carlton *et al.*, 1985

10. ***Related Proceedings Appendix.***

None

CONCLUSION

Appellants maintain that the Examiner has failed to carry her burden of establishing the claims are not patentable because she has (a) failed to establish a *prima facie* case of obviousness of claims 1 and 4-7 over Ben-Dov *et al.* in view of Carlton *et al*, Liu *et al.*, and deMaagd *et al.*,. and (b) failed to establish a *prima facie* case of obviousness of claims 2-3, 8-11, 19, 22-23, and 30 over Ben-Dov *et al.* in view of Carlton *et al*, Liu *et al.*, deMaagd *et al.*, and Koziel *et al.* Accordingly, claims 1-11, 19, 22-23, and 30 are allowable. For these reasons, presented in detail above, Appellants respectfully request the rejections be reversed.

It is not believed that extensions of time are required. However, in the event that extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR §1.136(a), and any fee required therefore is hereby authorized to be charged to Deposit Account No. 50-2510.

Respectfully submitted,

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Customer No. 95725 **BAYER CROPSCIENCE LP** **ATHENIX CORPORATION** 2 T .W. Alexander Drive Research Triangle Park, NC 27709 Tel (919) 549-2663 Fax Raleigh Office (919) 549-3994	ELECTRONICALLY FILED USING THE EFS-WEB ELECTRONIC FILING SYSTEM OF THE UNITED STATES PATENT & TRADEMARK OFFICE ON OCTOBER 17, 2011.

APPEALED CLAIMS

1. An isolated or recombinant nucleic acid molecule selected from the group consisting of:

a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3;

b) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or 4; and,

c) a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO:2 or 4, wherein said polypeptide has activity against lygus pests.

2. The isolated or recombinant nucleic acid molecule of claim 1, wherein said nucleotide sequence is a synthetic sequence that has been designed for expression in a plant.

3. The nucleic acid molecule of claim 2, wherein said synthetic sequence has an increased GC content relative to the GC content of SEQ ID NO:1 or 3.

4. A vector comprising the nucleic acid molecule of claim 1.

5. The vector of claim 4, further comprising a nucleic acid molecule encoding a heterologous polypeptide.

6. A host cell that contains the nucleic acid molecule of claim 1.

7. The host cell of claim 6 that is a bacterial host cell.

8. The host cell of claim 6 that is a plant cell.

9. A transgenic plant comprising the host cell of claim 8.

10. The transgenic plant of claim 9, wherein said plant is selected from the group consisting of maize, sorghum, wheat, sunflower, tomato, crucifers, peppers, potato, cotton, rice, soybean, sugarbeet, sugarcane, tobacco, barley, and oilseed rape.

11. A transgenic seed comprising the nucleic acid molecule of claim 1.

19. A method for producing a polypeptide with pesticidal activity, the method comprising culturing the host cell of claim 6 under conditions in which the nucleic acid molecule encoding the polypeptide is expressed.

22. A plant having stably incorporated into its genome a DNA construct comprising a nucleotide sequence that encodes a protein having pesticidal activity, wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3;

b) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or 4; and,

c) a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO:2 or 4, wherein said polypeptide has activity against lygus pests;

wherein said nucleotide sequence is operably linked to a promoter that drives expression of a coding sequence in a plant cell.

23. A plant cell having stably incorporated into its genome a DNA construct comprising a nucleotide sequence that encodes a protein having pesticidal activity, wherein said nucleotide sequence is selected from the group consisting of:

a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1 or 3;

b) a nucleic acid molecule which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:2 or 4; and,

c) a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having at least 95% amino acid sequence identity to the amino acid sequence of SEQ ID NO:2 or 4, wherein said polypeptide has activity against lygus pests;

wherein said nucleotide sequence is operably linked to a promoter that drives expression of a coding sequence in a plant cell.

30. The nucleic acid molecule of claim 1, wherein said nucleotide sequence is operably linked to a promoter capable of directing expression of said nucleotide sequence in a plant cell.

EVIDENCE APPENDIX A

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1996, p. 3140–3145 Vol. 62, No. 9
0099-2240/96/$04.00+0

Restriction Map of the 125-Kilobase Plasmid of *Bacillus thuringiensis* subsp. *israelensis* Carrying the Genes That Encode Delta-Endotoxins Active against Mosquito Larvae

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A large plasmid containing all delta-endotoxin genes was isolated from *Bacillus thuringiensis* subsp. *israelensis*; restricted by *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, and *Sal*I; and cloned as appropriate libraries in *Escherichia coli*. The libraries were screened for inserts containing recognition sites for *Bam*HI, *Sac*I, and *Sal*I. Each was labeled with ^{32}P and hybridized to Southern blots of gels with fragments generated by cleaving the plasmid with several restriction endonucleases, to align at least two fragments of the relevant enzymes. All nine *Bam*HI fragments and all eight *Sac*I fragments were mapped in two overlapping linkage groups (with total sizes of about 76 and 56 kb, respectively). The homology observed between some fragments is apparently a consequence of the presence of transposons and repeated insertion sequences. Four delta-endotoxin genes (*cryIVB-D* and *cytA*) and two genes for regulatory polypeptides (of 19 and 20 kDa) were localized on a 21-kb stretch of the plasmid; without *cytA*, they are placed on a single *Bam*HI fragment. This convergence enables subcloning of delta-endotoxin genes (excluding *cryIVA*, localized on the other linkage group) as an intact natural fragment.

Mosquitoes and blackflies are vectors of many human infectious diseases (22). One of the best biocontrol agents against their larvae is the bacterium *Bacillus thuringiensis* subsp. *israelensis* (serovar H14) (9, 20). Its mosquito larvicidal activity is included in five polypeptides of a parasporal crystalline body (delta-endotoxin [17, 20]), CryIVA-D and CytA (134, 128, 78, 72, and 27 kDa in size), encoded by genes which are highly expressed during sporulation (9). These, and all the other genetic elements responsible for toxicity, are located on one of the largest (125-kb) plasmids of *B. thuringiensis* subsp. *israelensis* (9, 16). The gene for a regulatory 20-kDa polypeptide (*p20*) which is required for efficient production of the structural proteins (25–27) is mapped downstream of *cryIVD* (1). A new open reading frame (*p19*) which encodes a putative 19-kDa polypeptide proposed as another chaperone has recently been found upstream of *cryIVD* (12). The DNA sequences indicate that *p19*, *cryIVD*, and *p20* form an operon (12). Several insertion sequences (IS*231*F, V, and W and IS*240*A and B) which seem to allow transposition, duplication, rearrangement, and modification have been found on this 125-kb plasmid (19).

The coding information known to date on the 125-kb plasmid accounts for less than 20% of its total length. The rest (over 80%), still unknown, may regulate expression of the structural genes. It is anticipated that deciphering the roles of other genes involved will enhance development of molecular procedures for improving mosquito biocontrol. A full restriction map will allow location of the toxin genes on the plasmid and improve our understanding of their interactions with other genes.

The plasmid has previously been mapped partially for *Bam*HI (15) and for *Sac*I as well (4). Here, it was completely mapped for *Bam*HI, *Sac*I, and *Sal*I and partially for *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I, and all eight currently known genes were localized on the derived map. The apparent convergence of most of them indeed enables subcloning of delta-endotoxin genes as an intact natural fragment.

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MATERIALS AND METHODS

Bacterial strains and plasmid vectors. Strain 4Q5 (original code, 4Q2-72), a derivative of *B. thuringiensis* subsp. *israelensis* cured of all its plasmids except the 125-kb one (8), was kindly supplied by D. H. Dean (Bacillus Genetic Stock Center, Columbus, Ohio) and served as the source for the 125-kb plasmid. Plasmids pUC-9 and pUC-19 were used as cloning vectors, and *Escherichia coli* XL-Blue MRF′ (Stratagene, La Jolla, Calif.), JM83, and JM109 were used as recipient strains.

Recombinant DNA methods. DNA modification and restriction enzymes were used as recommended by the suppliers (mostly New England BioLabs and United States Biochemical), and the procedures were carried out as described by Sambrook et al. (21). Competent cells were prepared and plasmids were isolated by standard procedures. Transformants of *E. coli* were selected on Luria broth plates containing ampicillin (50 to 100 μg ml^{-1}). For screening of recombinants, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or MacConkey plates were employed. Small-volume isolation of recombinant plasmids was performed by alkaline extraction (5). DNA was analyzed by electrophoresis on horizontal agarose slab gels and visualized with ethidium bromide. DNA fragments were purified from the gels by electrophoresis onto DEAE-cellulose membranes (21) or by phenol extraction (23).

Isolation of large plasmid DNA. The 125-kb plasmid DNA was isolated according to the method of Krens and Schilperoort (18) for the Ti plasmids of *Agrobacterium tumefaciens*. *B. thuringiensis* subsp. *israelensis* 4Q5 was grown in Luria-Bertani broth at 30°C. Cells were harvested in the early stationary phase and lysed with sodium dodecyl sulfate (1%) and pronase (0.25 mg ml^{-1}). The pH was raised to 12.2, at which chromosomal DNA but not plasmid DNA denatures, and then slowly lowered to 8.6. The suspension was brought to 1 M NaCl to precipitate denatured DNA and proteins, and the plasmid in the supernatant was precipitated by polyethylene glycol and purified by centrifugation in a CsCl-ethidium bromide gradient.

Southern blot hybridization. DNA of the 125-kb plasmid was digested with several restriction enzymes and subjected to 0.8 and 0.6% agarose gel electrophoresis for short and long runs, respectively. The DNA was denatured and transferred to nitrocellulose filters (Micron Separations, Westboro, Mass.) as described by Davis et al. (7), and conventional hybridization was carried out (21). Autoradiograms were obtained after appropriate exposure to X-ray films at −70°C.

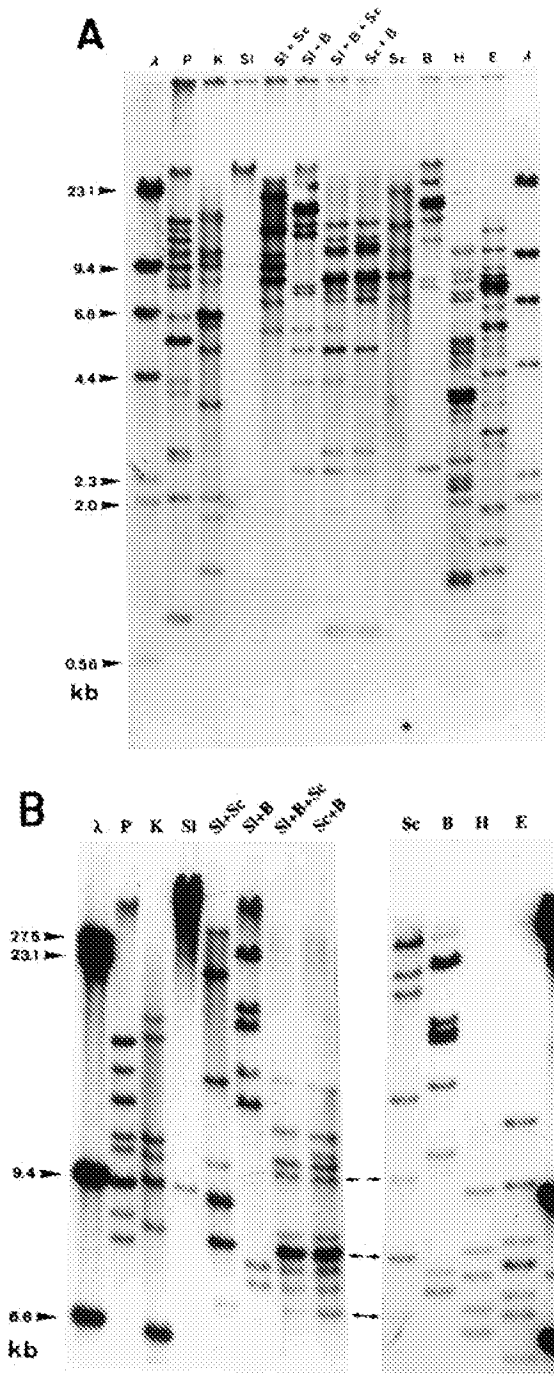

FIG. 1. Autoradiograms of Southern blots of the 125-kb plasmid (hybridized to ^{32}P-labeled plasmid itself as a probe), digested by the following restriction enzymes: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; Sc, *Sac*I; Sc+B, *Sac*I-*Bam*HI; Sl+B+Sc, *Sal*I-*Bam*HI-*Sac*I; Sl+B, *Sal*I-*Bam*HI; Sl+Sc, *Sal*I-*Sac*I; Sl, *Sal*I; K, *Kpn*I; and P, *Pst*I. λ, λ_{DNA} digest by *Hin*dIII. The blots are of short (15 h at 50 V) (A) and long (40 h at 40 V) (B) runs on agarose (0.8 and 0.6%, respectively) gels of the restriction digests. Panel B was assembled from two equivalent autoradiograms; internal arrows display identical bands.

RESULTS

Large DNA is more conveniently mapped by rare-cutting restriction enzymes than by frequent cutters. Three enzymes (*Bam*HI, *Sac*I, and *Sal*I) were chosen here because the GC content in their recognition sites is high (4 of 6 bases) while it is relatively low (ca. 35% [2]) in total DNA of *B. thuringiensis* strains.

TABLE 1. Sizes of fragments (derived from Fig. 1) of the 125-kb plasmid by restriction endonuclease digestion

Endonuclease(s)	Sizes (kb) of fragments generated	Total size (kb)
*Sal*I	~110, 9.4, 5.7	~125.1
*Sal*I-*Sac*I	31.0, 22.0, 14.0, 10.5, 9.4, 9.3, 8.5, (8.5?), 7.0, 5.7, 1.8, 0.4	128.1[a]
*Sal*I-*Bam*HI	27.5, 20.0, 18.0, 15.0, 13.5, 8.0, 7.5, 5.7, 5.0, 4.1, 2.5, 0.9	127.7
*Sal*I-*Bam*HI-*Sac*I	15.0, 12.5, 11.0, 10.5, 9.0, 8.5, (8.5?), 8.0, 7.5, 7.0, 5.7, 5.0, 5.0, 4.1, 2.7, 2.5, 1.8, 0.9, 0.7, 0.4	126.3[a]
*Sac*I-*Bam*HI	15.0, 12.5, 11.5, 11.0, 10.5, 9.0, 8.5, (8.5?), 8.0, 7.5, 7.0, 7.0, 5.0, 2.7, 2.5, 0.7, 0.4	127.3[a]
*Sac*I	31.0, 24.0, 22.0, 14.0, 10.5, 8.5, (8.5?), 7.0, 0.4	125.9[a]
*Bam*HI	27.5, 20.0, 18.5, 18.0, 15.0, 11.5, 8.0, 7.5, 2.5	128.5
*Pst*I	16.2, 15.0, 13.0, 11.2, 10.5, 9.3, 8.8, 8.2, 6.4, 5.4, 4.3, 4.2, 4.0, 2.8, 2.7, 2.1, 1.5, 0.82	126.4
*Kpn*I	17.5, 16.5, 11.5, 10.8, 9.4, 8.4, 6.6, 6.4, 5.8, 5.1, 3.8, 3.1, 2.8, 2.4, 2.1, 1.7, 1.2, 0.85	115.9
*Eco*RI	13.0, 10.2, 8.6, 8.0, 8.0, 7.4, 7.0, 6.3, 5.8, 5.7, 5.4, 4.8, 4.3, 3.5, 3.0, 2.8, 2.2, 1.9, 1.6, 1.3, 0.78, 0.13, 0.08	111.8
*Hin*dIII	9.7, 8.4, 7.9, 7.2, 6.8, 5.4, 5.2, 5.0, 4.7, 4.5, 4.1, 4.0, 3.9, 3.7, 3.4, 3.2, 2.6, 2.4, 2.3, 2.1, 1.8, 1.7, 1.5, 1.3, 1.2, 1.0, 0.82, 0.66, 0.48	107

[a] Includes two *Sac*I 8.5-kb fragments, one of which is indicated in the second column of data by a question mark in parentheses.

Preliminary data. The plasmid (125 kb) containing all genetic information required for toxicity was restricted by *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, and *Sal*I, singly or in four

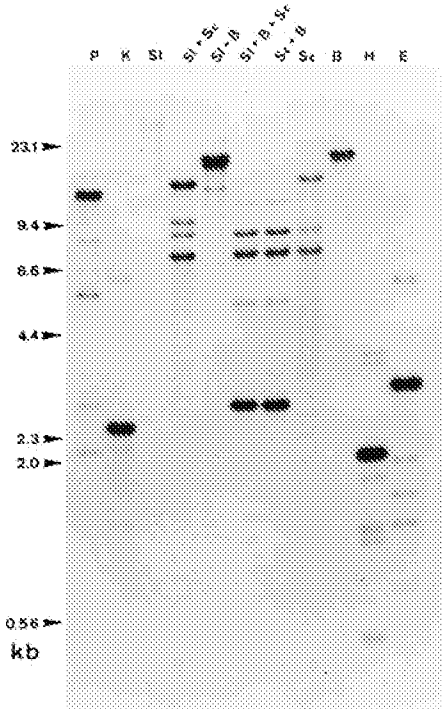

FIG. 2. Autoradiogram of a Southern blot (same as in Fig. 1A) hybridized to the ^{32}P-labeled *Kpn*I 2.4-kb fragment as a probe (Table 2).

TABLE 2. Sizes of plasmid fragments detected by hybridization to eight different probes

Probe[a]	Size(s)[b] of fragments										
	*Sal*I	*Sal*I-*Sac*I	*Sal*I-*Bam*HI	*Sal*I-*Bam*HI-*Sac*I	*Sac*I-*Bam*HI	*Sac*I	*Bam*HI	*Pst*I	*Kpn*I	*Hin*dIII	*Eco*RI
*Kpn*I-2.0 kb (B)	>50	(9.3)	13.5	11, 2.7, (5)	11, 2.7, (5)	14, (8.5)	20, 18.5, 18	13, (5.4)	2.0, (3.8)	2.1, (3.7)	8.0
*Kpn*I-2.4 kb (Sc,Sc)	>50	(9.3)		2.7	2.7	14, 0.4, 7, (8.5)	20	13	2.4	2.1, (0.66)	3.0
*Kpn*I-5.1 kb (Sc)	>50			12.5	12.5	22, 10.5	27.5	10.5, 4.2	5.1	5, 2.3, 1.2, (1.5, 0.48)	4.8, 2.2
*Kpn*I-5.8 kb (B)	>50					30	15, 7.5	8.8, (11.2, 1.5)	5.8	3.7, 2.4, (2.1)	7, 3.5, (13)
*Kpn*I-6.4 kb (B, Sc)	>50			0.7, (5)	0.7, (5)	22, 8.5, (14)	18, 2.5	9.3, 5.4, (0.82)	6.4	4.1, (1.2)	7.4
*Kpn*I-8.4 kb (B, Sl)	9.4	1.8	13.5, 5, 4.1	5, 4.1, 1.8	7	24	18.5, 11.5, (27.5)	16.2, 15, (10.5)	8.4	7.9, 3.7, (9.7)	8.6, 4.3, 3
*Hin*dIII-9.7 kb (B)	(9.4)		5	12.5, 5	12.5	22	27.5, 8, (18.5)	10.5, 9.3	16.5, 8.4	9.7, (7.9)	5.4, 4.3, 3, 0.8
*Pst*I-11.2 kb (B)	>50			9.0	9.0	31	20, 7.5, (15)	11.2, (8.8)	11.5, 2.8, (17.5)	7, 2.1, (4)	13, 7, (8)

[a] Fragment used as the probe, with at least one recognition site for *Bam*HI (B), *Sac*I (Sc), or *Sal*I (Sl) (indicated in parentheses).
[b] Numbers in parentheses designate fragments weakly hybridized, indicating partial homologies to probes.

FIG. 3. Autoradiogram of a Southern blot (same as in Fig. 1A) hybridized to the ^{32}P-labeled *Kpn*I 5.1-kb fragment as a probe (Table 2).

combinations as follows: *Sal*I-*Bam*HI-*Sac*I, *Sac*I-*Bam*HI, *Sal*I-*Bam*HI, and *Sal*I-*Sac*I. The products were separated on agarose gels, and their blots were hybridized to the radiolabeled (nick-translated) plasmid itself (Fig. 1; Table 1). Two relatively small fragments (5.7 and 9.4 kb) were generated by *Sal*I, and the sizes of the rest could not be determined under the conditions employed. Nine fragments (two of which were separable by long runs only on agarose gels), summing up to about 128 kb, were generated by *Bam*HI, and eight fragments, summing up to about 118 kb, were generated by *Sac*I. This apparent discrepancy can be resolved if two fragments of *Sac*I with similar sizes (ca. 8.5 kb) overlap in the gel, as indicated by the higher intensity of the *Sac*I 8.5-kb band than those of the *Sac*I 7- and *Sac*I 10.5-kb bands (Fig. 1). In addition, the sum of the sizes of the fragments generated by digestion with the four combinations (as above) ranged between 125 and 128.5 kb (Table 1). Digestion with the frequent cutters, *Eco*RI, *Hin*dIII, *Kpn*I, and *Pst*I, yielded summed sizes between 107 and 126 kb (Table 1), negatively correlated to the number of fragments generated.

The four largest *Bam*HI fragments (18, 18.5, 20, and 27.5 kb) were cut once each by *Sac*I, and the four largest *Sac*I fragments (14, 22, 24, and 31 kb) were cut once each by *Bam*HI (Fig. 1; Table 1). The double digest yielded seven *Sac*I-*Bam*HI fragments (12.5, 11, 9, 7, 5, 2.7, and 0.7 kb). Two *Bam*HI fragments (11.5 and 18.5 kb) were cut by *Sal*I, and two *Sal*I fragments (9.4 kb and the large one) were cut by *Bam*HI. These double digests yielded four *Sal*I-*Bam*HI fragments (13.5, 5, 4.1, and 0.9 kb). Only one *Sac*I fragment (24 kb) was cut by *Sal*I to generate two *Sac*I-*Sal*I fragments (9.3 and 1.8 kb). The 13.5-kb *Bam*HI-*Sal*I fragment was cut by *Sac*I, and the 9.3-kb *Sac*I-*Sal*I

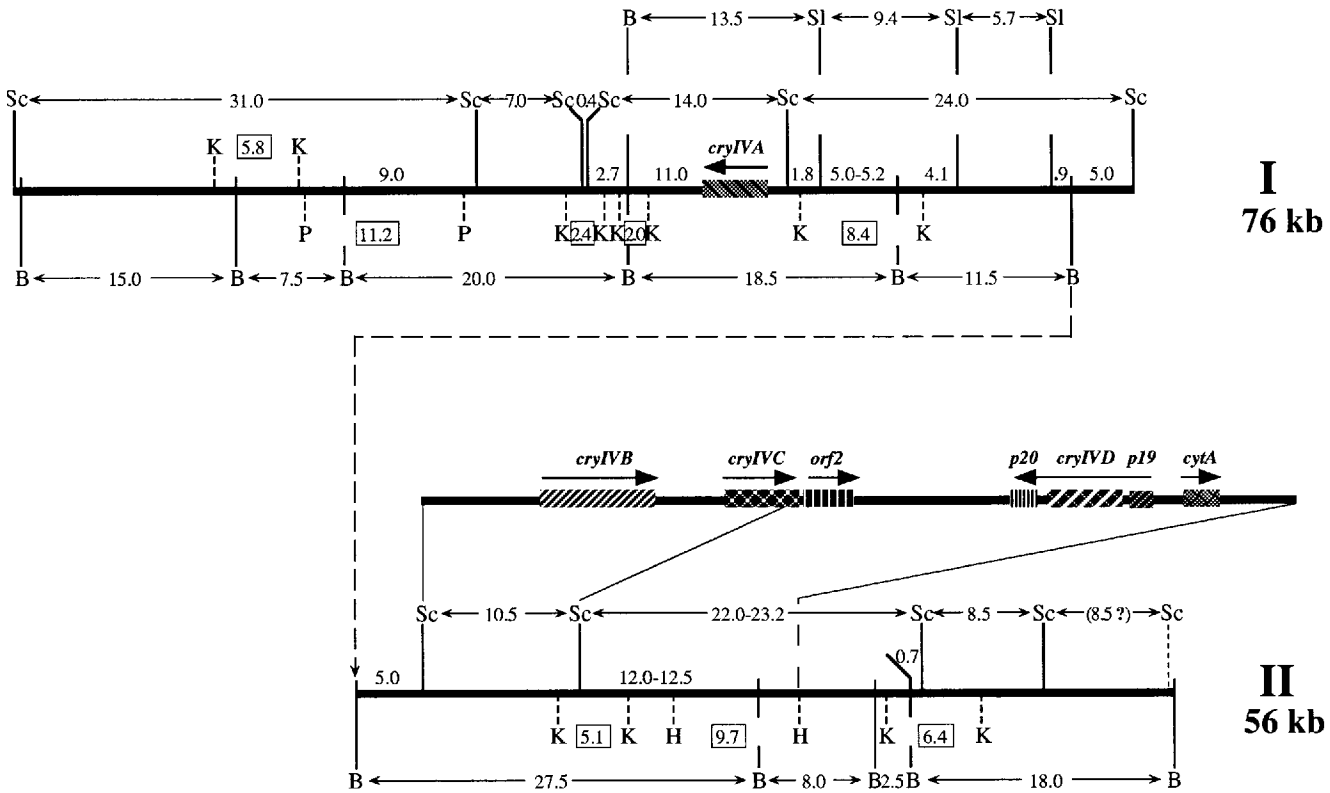

FIG. 4. Partial physical map of the *B. thuringiensis* subsp. *israelensis* H-14 125-kb plasmid. Numbers indicate sizes (in kilobases) of the relevant fragments, some of which (*Bam*HI [B], *Sac*I [Sc], *Sal*I [Sl], and one *Bam*HI-*Sal*I [B-Sl]) are enclosed by double-headed, thin arrows and others of which (those of the probes) are boxed. Other abbreviations are defined in the Fig. 1 legend. The broken arrow displays the overlap site between the two linkage groups (I, of 76 kb; II, of 56 kb). Genes are indicated by hatched boxes, and their transcription directions are indicated by thick arrows. The 26-kb (*Sac*I-*Sac*I-*Hin*dIII) region with most of the known genes is enlarged about 2.3-fold.

fragment was cut by *Bam*HI (triple digest in Fig. 1 and Table 1).

Mapping by hybridization to selected probes. The 125-kb plasmid was restricted by *Hin*dIII, *Kpn*I, and *Pst*I and cloned as appropriate libraries in *E. coli*. The libraries were screened for inserts containing recognition sites for *Bam*HI, *Sac*I, or *Sal*I. Radiolabeled inserts with at least one of these recognition sites each were exploited as probes for hybridization to Southern blots (same as in Fig. 1) to align at least two of the relevant fragments at a time. For example, the labeled *Kpn*I 2.4-kb insert (containing two *Sac*I sites, positioned 0.4 kb apart) detected three *Sac*I bands (0.4, 7, and 14 kb long), a single (20-kb) *Bam*HI band, and a 2.7-kb *Bam*HI-*Sac*I band (Fig. 2; Table 2). This information linked the three *Sac*I fragments to the single *Bam*HI 20-kb fragment (see Fig. 4).

The 5.1-kb *Kpn*I probe (containing one *Sac*I site) similarly linked two *Sac*I fragments (10.5 and 22 kb long), a single (27.5-kb) *Bam*HI fragment, and a 12.5-kb *Bam*HI-*Sac*I fragment (see Fig. 4): it hybridized to the respective bands (Fig. 3; Table 2).

An interesting, crucial probe for the mapping (Fig. 4) is the 8.4-kb *Kpn*I probe: in addition to a *Bam*HI site, it contains one of the only three *Sal*I sites of the plasmid (Fig. 1; Table 1). This probe hybridized to the following bands (Table 2): two *Bam*HI bands (18.5 and 11.5 kb), one *Sac*I band (24 kb), two *Sal*I bands (9.4 and 5.7 kb), three *Bam*HI-*Sal*I bands (13.5, 5, and 4.1 kb), one *Sac*I-*Sal*I band (1.8 kb), and one *Sac*I-*Bam*HI band (7 kb).

Table 2 summarizes the data obtained from these and five additional probes. Together with the preliminary data, they allowed construction of two linkage groups (of 76 and 56 kb) (Fig. 4).

Gene localization and homologies. Hybridization results were not always simple to interpret because of gene duplications and insertion sequences, which had been documented before (6, 19); they indeed hampered map construction. However, once a reasonable map has been assembled (Fig. 4), it became evident that a consistent picture could be obtained. For example, the genes *cryIVA* and *cryIVB*, which are highly homologous, had been mapped on the *Sac*I 14- and 10-kb fragments, respectively (6). When *cryIVA* (on a PCR-derived 3.5-kb DNA [3]) was used as a probe, it indeed detected them both (Fig. 5), as well as the two overlapping *Bam*HI fragments (27.5 and 18.5 kb), confirming the map of Fig. 4.

Four genes which had previously been positioned on the *Hin*dIII 9.7-kb fragment (1, 12–14) were localized on the plasmid relative to the *Bam*HI and *Sac*I maps and to the other genes in this work (Table 2; Fig. 4; see also Discussion).

DISCUSSION

Genes for delta-endotoxin, beta-exotoxin, and thuricin are clustered on either the same or different replicons, usually very large plasmids or the chromosome itself, in various *B. thuringiensis* subspecies (19). *B. thuringiensis* subsp. *israelensis* (serovar H14) is an exclusively delta-endotoxin producer (9). All genes necessary for synthesis of its toxin are located on a single, high-molecular-weight plasmid of about 125 kb (9, 16).

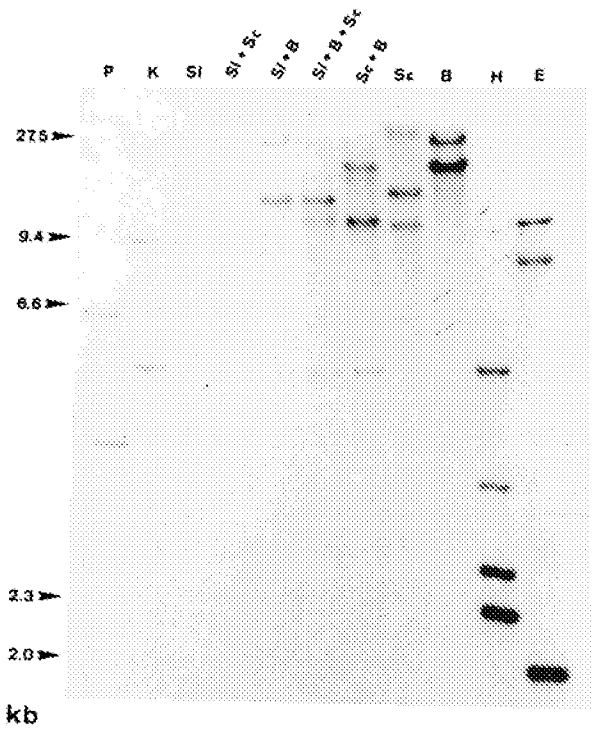

FIG. 5. Autoradiogram of Southern blot (same as in Fig. 1A) hybridized to ^{32}P-labeled PCR-derived *cryIVA* on a 3.5-kb fragment as a probe (Table 2).

The plasmid was partially mapped in this study (Fig. 4) through analysis of products obtained by cleavage with several restriction endonucleases (Fig. 1; Table 1) and hybridization to reference fragments (Fig. 2, 3, and 5; Table 2). The two linkage groups (of 76 and 56 kb) thus constructed (Fig. 4) can logically be joined by the 5-kb *Bam*HI-*Sac*I fragment at the end of each, which seem to overlap. Moreover, it is the only *Sac*I-*Bam*HI fragment that was detected by none of the probes except itself. Construction of a consistent, circular map of the plasmid was obtained by assuming that the second *Sac*I 8.5-kb fragment, proposed in Results, "Preliminary data," is found at the end of the 18-kb *Bam*HI fragment.

Ambiguous results were obtained with several additional probes (data not shown), which could be attributed to the known multiple insertion sequences (IS*231*F, V, and W and IS*240*A and B [1, 11, 19]) and homologous regions (17) on the plasmid. A case in point which could be resolved is the known homology between *cryIVA* and *cryIVB*; they had been mapped on *Sac*I 14- and 10-kb fragments, respectively (6). Labeled *cryIVA* indeed detected both of them, as well as the two overlapping *Bam*HI fragments (18.5 and 27.5 kb) (Fig. 5). In connection, it should be mentioned that the *Sac*I 10-kb fragment contains, in addition to *cryIVB*, most of *cryIVC*. The conclusion (6) that this fragment includes three copies of *cryIVB* becomes very unlikely, considering that most of its length is occupied by these two genes and the stretch between them (10). More data are needed before any conclusions can be drawn about possible interactions between these genes from their proximity on this *Sac*I 10.5-kb fragment.

The *Hin*dIII 9.7-kb fragment (1) was used as a probe (Table 2) to join two *Bam*HI fragments, 8 and 27.5 kb long. Its *Bam*HI site splits *cytA* (1), and three additional genes, *p19*, *cryIVD*, and *p20* (as well as IS*231*W), were located on the larger half (1, 12). These four genes, and the known open reading frame *orf2* (10, 24), are all included on the *Sac*I 22-kb fragment.

It is instructive that seven of the eight known genes (excluding *cryIVA*) were located on a 21-kb section of the plasmid (linkage group II) (Fig. 4). Without *cytA*, they are placed on a single (27.5-kb) *Bam*HI fragment. This convergence enables subcloning of most delta-endotoxin genes as an intact natural fragment.

The gene *cryIVA* is at least 27 kb removed from the cluster of the other seven known genes (Fig. 4). It is flanked by two symmetrical IS*240* sequences (A and B) of 865 bp each on the *Sac*I 14-kb fragment (6, 11). The genes on the stretch of 6 kb downstream of *cryIVA* inside this region are still to be determined.

The plasmid map derived in this study (Fig. 4) will be helpful for discovering new delta-endotoxin genes, localizing them relative to the currently known genes, and understanding interactions between them and with adjacent regulatory elements such as promoters, enhancers, terminators, stabilizers, and mobile elements. It would be indispensable for the ultimate description of the plasmid and for relating nucleotide sequences to the entire genome.

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EVIDENCE APPENDIX B

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Plasmids and Delta-Endotoxin Production in Different Subspecies of *Bacillus thuringiensis*

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Natural isolates of *Bacillus thuringiensis* have been classified into over 20 subspecies (varieties) and subvarieties (28 to date) according to their flagellar serotypes (H-antigens) and other biochemical properties (1). Preparations of *B. thuringiensis* spores and crystals have been used agriculturally as bioinsecticides against larvae of lepidopteran pests and, more recently, against some dipteran larvae (4). The insecticidal activity resides in the proteinaceous inclusion, generally bipyramidal, that is formed during sporulation and is known as the parasporal body or crystal; the protein toxin itself is known as the delta-endotoxin (7). Plasmids are ubiquitous in *B. thuringiensis* strains, usually in complex arrays (B. C. Carlton and J. M. González, Jr., *in* D. Dubnau, ed., *The Molecular Biology of the Bacilli*, vol. 2, in press), and some of these plasmids are capable of transfer between *B. thuringiensis* strains and into *B. cereus* (10). Delta-endotoxin genes have been assigned to specific *B. thuringiensis* plasmids by studies involving isolation of acrystalliferous (Cry^-) *B. thuringiensis* variants by plasmid curing (12, 23), cloning of toxin genes (14, 21), and transfer of toxin plasmids into Cry^- *B. thuringiensis* and *B. cereus* strains, which were thereby converted into crystalliferous (Cry^+) toxin producers (9).

Intravarietal similarities among the complex *B. thuringiensis* plasmid arrays were suggested in reports on different isolates of the same subspecies (12, 14). A general survey of the plasmid content of all *B. thuringiensis* subspecies would be useful as a first step towards identifying new transmissible toxin plasmids, which might be combined in a single *B. thuringiensis* recipient to generate strains with multiple toxin plasmids (9). Furthermore, it might prove feasible to use some of the more complex plasmid patterns as an aid to classification of *B. thuringiensis* strains. Toward these ends, the plasmid arrays of *B. thuringiensis* strains representing every subspecies were visualized and compared.

MATERIALS AND METHODS

Most of the *B. thuringiensis* strains were obtained from the collection of H. T. Dulmage, Cotton Insects Research, U.S.D.A.-S.E.A., Brownsville, Tex., and carry serial numbers with the prefix "HD-." The catalog of HD strains (5) contains information on strains available and their sources. A few strains were obtained from H. deBarjac (Institut Pasteur, Paris), D. H. Dean (Ohio State University, Columbus), and A. J. DeLucca II (Southern Regional Research Center, U.S.D.A., New Orleans, La.).

Plasmid patterns were visualized on 0.5% vertical agarose gels using Tris-borate running buffer and a high running voltage, by our modification (9) of the method of Eckhardt (6). Sizes of unknown plasmids were estimated by comparison with the plasmids of strain HD-2, which were measured previously by electron microscopy (12).

RESULTS AND DISCUSSION

Plasmid arrays of *B. thuringiensis* strains. Strains representing all known *B. thuringiensis* subspecies, including the recent isolates of serotypes 17 (19), 18 and 19 (20), 20 (22), and 21 (3), were examined for plasmid DNA. As shown in Fig. 1, all strains contained plasmid DNA ranging from 1 to 12 plasmids per strain. Generally, the plasmid array of each *B. thuringiensis* serotype or subspecies was unique and could be distinguished from that of other strains, although there were cases of internal diversity of plasmid content within serotypes. Some new examples of strains carrying plasmids that were present mostly in the open circular (OC) or linear form (12) were also found.

The results in Fig. 1a are typical of the variations in plasmid number and size range observed. The plasmids range from 2 to 12 per strain and from ~1.4 to ~150 megadaltons (MDa) in size. There are no obvious resemblances in plasmid arrays among the subspecies represented. When strains of the same subspecies are compared, however, such as HD-4 and *anduze* (both subsp. H-3a *alesti*), similarities in plasmid content are obvious. The five largest plasmids in HD-4 and *anduze* are of identical sizes. The smaller plasmids are less similar, but still rather alike. In the three H-3ab *kurstaki* strains, however, the small plasmids are similar and the large plasmids are different. Figure 1a also shows a

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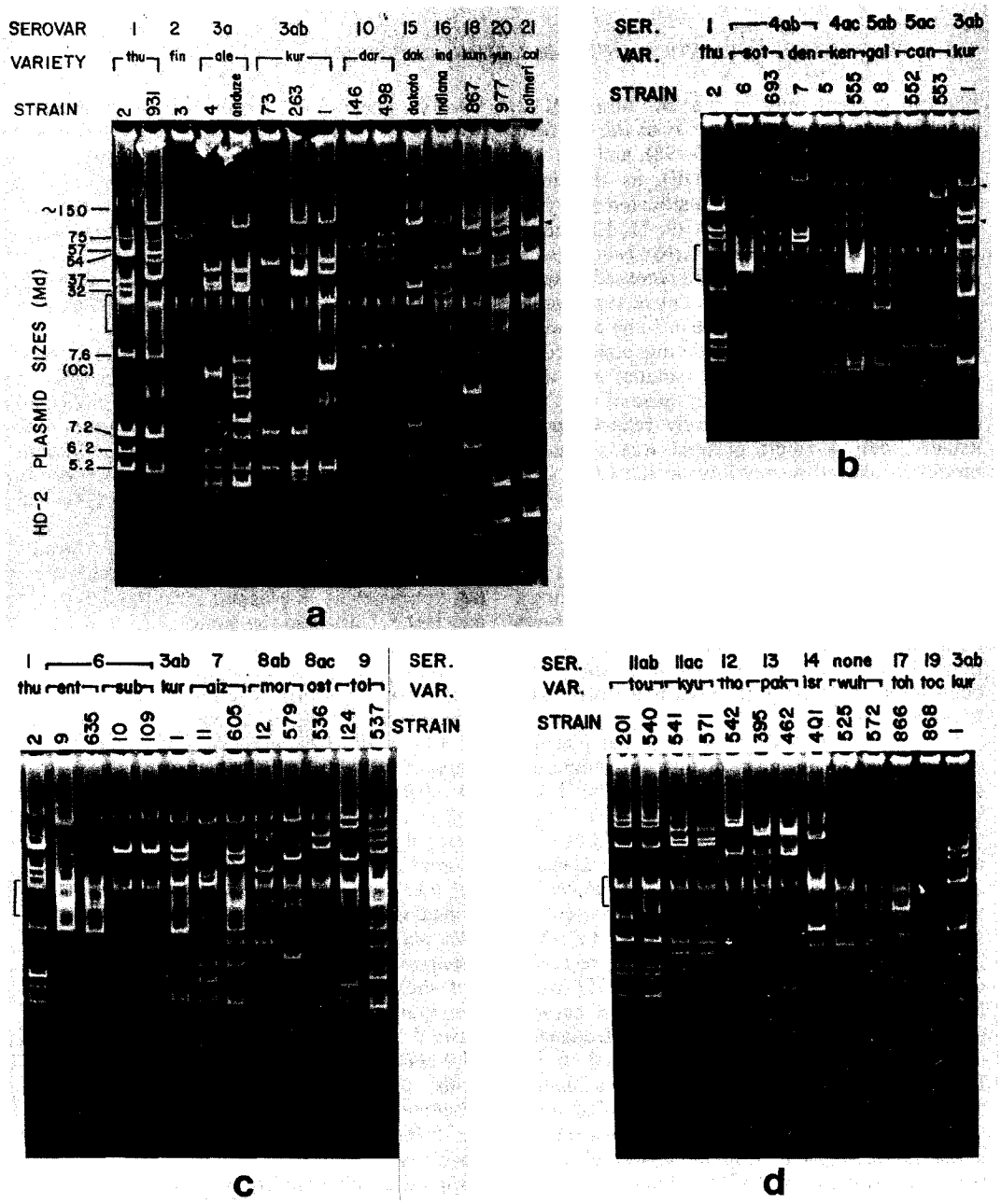

FIG. 1. Modified Eckhardt lysate-electrophoresis (9) on 0.5% agarose of cells of representative strains of *B. thuringiensis*, demonstrating the presence of complex plasmid arrays in most of them. The subspecies represented are as follows. (a) H-1, *thuringiensis* (thu); H-2, *finitimus* (fin); H-3a, *alesti* (ale); H-3ab, *kurstaki* (kur); H-10, *darmstadiensis* (dar); H-15, *dakota* (dak); H-16, *indiana* (ind); H-18, *kumamotoensis* (kum); H-20, *yunnanensis* (yun); H-21, *colmeri* (col). Serovar (serotype), subspecies, and strain designations are listed above each gel lane. The masses of the HD-2 plasmids, listed on the left margin, are intended as size standards. A broad band corresponding to linearized plasmids and chromosomal fragments is present in each lane; its general position is indicated by the bracket on the left margin. The open circles (O) indicate some of the plasmids (such as those in HD-2 and HD-4) which are present mostly or only in the OC form; the (L) indicates the linear plasmidlike DNA element (LDE), ~10 MDa in size, present in HD-1 and other strains below. The arrowheads indicate individual plasmids implicated in toxin production in the course of our studies. (b) Subspecies H-4ab *sotto* (sot) and *dendrolimus* (den); H-4ac *kenyae* (ken); H-5ab *galleriae* (gal); H-5ac *canadensis* (can); HD-2 and HD-1 are included for comparison. Symbols are as in (a). (c) Subspecies H-6 *entomocidus* (ent) and *subtoxicus* (sub); H-7 *aizawai* (aiz); H-8ab *morrisoni* (mor); H-8ac *ostriniae* (ost); H-9 *tolworthi* (tol); HD-2 and HD-1 are included for comparison. Symbols are as in (a). (d) Subspecies H-11ab *toumanoffi* (tou); H-11ac *kyushuensis* (kyu); H-12 *thompsoni* (tho); H-13 *pakistani* (pak); H-14 *israelensis* (isr); *wuhanensis* (wuh); H-17 *tohokuensis* (toh); H-19 *tochigiensis* (toc). HD-1 is included for comparison; symbols are as in (a).

plasmid present only in the OC form (HD-2, HD-931), several present mostly as the OC form (HD-4, *anduze*, HD-146, HD-498), and one, the "linear DNA element" (LDE), as the linear form (HD-1). The plasmids implicated in crystal toxin production in our work (9, 11, 12; unpublished data) are indicated by arrowheads. Isolation of single colonies from the received cultures sometimes aided the assignment of toxin genes to a specific plasmid, because in some cultures a significant part of the population consisted of Cry^- variants. These were isolated and their plasmids were compared with those of the Cry^+ cells of the same or a closely related strain; usually, only a single plasmid was absent or modified (altered in mobility) in the Cry^- variant. Most of these plasmids encode a phase-refractile crystal under most standard media and conditions, such as NSM agar (16) at 30°C; two of them, the HD-2 54-MDa plasmid and the HD-1 44-MDa plasmid are unusual in encoding a crystal only in certain media or temperatures (González and Carlton, manuscript in preparation). Individual *B. thuringiensis* strains have been found to carry one, two, and even three toxin plasmids.

Figure 1b further demonstrates that plasmid patterns can be used to distinguish individual strains of the same flagellar serotype. The H-4ab strains HD-693 (biotype *sotto*) and HD-7 (biotype *dendrolimus*) have distinct plasmid arrays, as do HD-5 and HD-555 (both H-4ac *kenyae*, one from Kenya, the other from India). Plasmids present mostly as the OC form are present in strains HD-5 (H-4ac *kenyae*) and HD-8 (H-5ab *galleriae*). It is interesting that the received cultures of HD-6, HD-552, and HD-553 were found to be totally Cry^-. The loss of crystal production in HD-6 might be linked with changes in the single plasmid seen in the related strain, HD-693, which is present in HD-6 is in a smaller (deleted?) form. The single plasmid of a *sotto* strain (probably identical to HD-693) was implicated in toxin production by Kronstad et al. (14). The tendency of H-5ac *canadensis* strains to become Cry^- upon subculturing has been previously reported (2).

Figure 1c demonstrates the presence of complex plasmid arrays in *B. thuringiensis* serotypes H-6 through H-10. HD-9 and HD-635 are derivatives of a single original isolate (H. T. Dulmage, personal communication). HD-9 makes large, blunt bipyramidal crystals with pronounced toxicity against *Aedes aegypti* larvae, and HD-635 makes tiny crystals, nontoxic to *A. aegypti* (unpublished data), and also lacks the ~105- and 29-MDa plasmids present in HD-9 (the other large plasmid and LDE appear to be present in both strains). Derivatives of HD-9 (not shown) have been isolated that lack the 29-MDa plasmid but retain their large, mosquito-toxic crystals, so that it can be deduced that the ~105-MDa plasmid of HD-9 is involved in delta-endotoxin production. HD-9 may also carry a second toxin plasmid since HD-635 still produces (tiny) crystal inclusions. The presence of LDE molecules with the same mobility as the LDE of HD-1 is evident in HD-6, HD-635, HD-605 (H-7 *aizawai*), and HD-537 (H-9 *tolworthi*).

Figure 1d shows several pairs of *B. thuringiensis* strains, most of them from a single original isolate, which have begun to diverge in plasmid content. HD-201 and HD-540 are from the same isolate, but during passage through different laboratories, their OC plasmids have diverged in size. Likewise, part of the population of HD-395 retains the same plasmid content as HD-462, whereas part has started to mutate. Strains HD-541 and HD-571, on the other hand, have not yet diverged in plasmid array, and HD-525 and HD-572 are also the same.

All strains of H-14 *israelensis* (*B. thuringiensis* subsp. *israelensis*) examined had nearly identical plasmid patterns; the differences could be explained as simple loss of one or more plasmids from the single original isolate of Goldberg and Margalit (8), which was the source of ONR-60A, 4Q1, 4Q2, HD-500, HD-567, and other *B. thuringiensis* subsp. *israelensis* strains (D. H. Dean, personal communication). Comparison of the plasmid arrays of these strains suggested that 4Q1, the strain shown in Fig. 1d, is closest to the original isolate. Presumably, the loss of a ~125-MDa plasmid (present in 4Q1) produced the *B. thuringiensis* subsp. *israelensis* strain HD-567 (not shown), in which a 75-MDa plasmid has been implicated in toxin production by means of plasmid curing and plasmid transfer (11). Loss of a 4.9-MDa plasmid (present in 4Q1) probably led to the plasmid array of HD-500, another *B. thuringiensis* subsp. *israelensis* strain in which the 75-MDa plasmid has been implicated in toxin synthesis by curing (1).

Figure 1d also shows two cultures of strain *wuhanensis*, a nonmotile *B. thuringiensis* strain from China. Their plasmid arrays are the same and are also almost identical to that of HD-8 (H-5ab *galleriae*) in Fig. 1b. This close resemblance suggests a common origin for H-5ab *galleriae* and the *wuhanensis* isolates. Finally, the plasmids of H-17 *tohokuensis* (HD-866) and H-19 *tochigiensis* (HD-868) strains are shown. HD-868 is unusual in possessing only a single plasmid, of rather large size (>100 MDa).

The number and variety of each strain in which a plasmid has been implicated in toxin production, and the size of the plasmid, are listed in Table 1. It is evident from Table 1 that all plasmids implicated in toxin production in

TABLE 1. Correlations between specific plasmids and delta-endotoxin production in various strains of *B. thuringiensis*

Prototype strain no.[a]	H-serotype (flagellar antigens) and subspecies	No. of plasmids	Size of plasmid (MDa) implicated in toxin production[b]	Type of implicating evidence (and references)[c]
HD-2	1, *thuringiensis*	10	75	A, T (1, 9, 12)
			[54]	T (González and Carlton, in preparation)
HD-225	1, *thuringiensis*	11	~110 (P1, P2)	D (González and Carlton, in preparation)
HD-73	3ab, *kurstaki*	6	50	A, T (9, 12)
HD-74	3ab, *kurstaki*	7	55	A, T (1; González and Carlton, in preparation)
HD-1	3ab, *kurstaki*	12	~115 (P1, P2)	A, D (González and Carlton, in preparation)
			[44 (P1)]	A, T (González and Carlton, in preparation)
HD-263	3ab, *kurstaki*	11	~110 (P1, P2)	A (González and Carlton, in preparation; Yamamoto et al., in preparation)
			60 (P1)	A, I (González and Carlton, in preparation)
			44 (P1)	T (9)
HD-4	3a, *alesti*	10	~105	A (12)
HD-8	5ab, *galleriae*	4	~130	D (12)
HD-9	6, *entomocidus*	4	~105	A (this study)
HD-536	8ac, *ostriniae*	4	68	A, T (1; this study)
HD-13	9, *tolworthi*	7	~110	A (González and Carlton, in preparation)
			44	A (González and Carlton, in preparation)
HD-146	10, *darmstadiensis*	5	70	A (this study)
HD-498	10, *darmstadiensis*	5	65	A (this study)
HD-542	12, *thompsoni*	4	~100	A (1)
HD-567	14, *israelensis*	9	75	A, T (11)
HD-500	14, *israelensis*	9	75	A (1)
BT Col	21, *colmeri*	6	~100	D (this study)

[a] Refer to numbers in the strain collection of H. T. Dulmage, who provided the original cultures, except for BT Col, the *B. thuringiensis* subsp. *colmeri* isolate, provided by A. J. DeLucca II.

[b] Plasmids in brackets code for crystal synthesis conditionally only (dependent on growth medium, or temperature, or both). P1 and P2 distinguish between plasmids coding for P1 toxin only and plasmids encoding both P1 and P2 toxins, in strains that produce both types of crystal (González and Carlton, in preparation; Yamamoto et al., in preparation).

[c] A, Absence of the plasmid in Cry^- variant(s); D, deleted form of the plasmid detected in Cry^- variant(s); I, form of plasmid with insertion detected in Cry^- variant(s); T, transfer of the plasmid into a Cry^- *B. thuringiensis* or *B. cereus* recipient converted it to Cry^+.

our studies were invariably large (>40 MDa). No plasmid <40 MDa has yet been implicated. Other laboratories have also implicated large *B. thuringiensis* plasmids (14, 23), but no small plasmid has been convincingly associated with production of crystal toxin (Carlton and González, in press).

Plasmid-toxin association in HD-1. Strain HD-1 (H-3ab, *kurstaki*), the strain of commercial importance in the United States, has also proved to be interesting scientifically. Our initial study (12) suggested that a 29-MDa plasmid was involved in toxin production in HD-1; this conclusion was based on isolation of a single Cry^- mutant (HD1-25), in which the 29-MDa plasmid had acquired an insertion of 3 to 4 MDa of DNA. Further analysis of this strain and of other HD-1 derivatives has led us to conclude that the 29-MDa plasmid does not code for the toxin crystal and that instead a large (~115-MDa) plasmid is involved in toxin crystal production. Some representative variants of HD-1 are discussed here, both as an example of the misleading results that may occur during genetic study of *B. thuringiensis* and of how *B. thuringiensis* plasmid arrays may change over time.

Figure 2 shows the plasmid patterns of the prototype strain, HD1-1, and of several derivatives, both Cry^+ and Cry^-, having altered plasmid arrays. HD1-1 lost the 44-, 51-, 9.6-, and ~130-MDa plasmids spontaneously (in four sequential steps; the intermediate strains, not shown, were Cry^+) to generate the Cry^+ variant HD1-14. HD1-14 then apparently lost the ~115-MDa plasmid to produce the Cry^+ variant HD1-15. However, HD1-15 proved unusual in that it generated several variants, both Cry^+ and Cry^-, containing new plasmids. One of them was HD1-18, in which a very faint band of ~160-MDa (not visible in Fig. 2) appeared.

HD1-18 then mutated to give the Cry$^-$ variant HD1-25, in which the ~160- and 29-MDa plasmids were replaced by plasmids of ~105 and 34 MDa. Other derivatives of HD1-15 harboring new plasmids are HD1-19, HD1-20, and HD1-21. HD1-19 is Cry$^+$ and is possibly a true "revertant," since its new plasmid is ~115 MDa. HD1-20 is Cry$^-$ and has a new plasmid of 88 MDa, whereas HD1-21 is Cry$^+$ and has a new 80-MDa plasmid. A line of descent entirely separate from that leading to HD1-14 produced the variants HD1-7 and HD1-9. Strain HD1-1 generated HD1-7 (Cry$^+$) through loss of the LDE and the 51-, 5.4-, 9.6-, and ~130-MDa plasmids, some by growth at 37 or 42°C and some by spontaneous curing. The strains representing the four intermediate stages are not shown. Growth of HD1-7 at 42°C generated the Cry$^-$ mutant HD1-9, which has lost the ~115-MDa plasmid.

In our earlier study of HD-1 (12), we believed that the ~115-MDa plasmid had been lost in HD1-15; cells of the HD1-19 type were detected in the HD1-15 culture but were thought to be contaminants. We now believe that the ~115-MDa plasmid was not lost in HD1-15 since new plasmids, possibly derived from it, predictably arise in the HD1-15 cell population, a phenomenon not seen in other variants of HD1-1. Some of the new plasmids are larger than the original ~115-MDa plasmid; others are smaller and may be deleted versions. Some of the strains with new plasmids are Cry$^+$ and some are Cry$^-$. New plasmids smaller than ~115 MDa are not necessarily associated with the Cry$^-$ phenotype since the Cry$^+$ variant HD1-21 contains a new 80-MDa plasmid that is smaller than the new 88-MDa plasmid of (Cry$^-$) HD1-20. Strain HD1-15 is unstable and generates spontaneous Cry$^-$ mutants (such as HD1-20) at a detectable frequency of 0.1 to 1%, much higher than the rate of mutation to Cry$^-$ in other HD-1 variants, where it is 0.01% or less. In contrast to the Cry$^-$ mutants derived from HD1-15, the Cry$^-$ mutant HD1-9 appears to have arisen by simple loss of the ~115-MDa plasmid.

Our new data implicate the ~115-MDa plasmid in crystal production in HD-1. The unusual variant HD1-15 may be analogous to the HD-567 variant HD567-26 (11), in that the probable toxin plasmid is undetectable on gels but remains present and is capable of generating smaller versions of itself, as shown in Fig. 2. We also conclude that the insertion into the 29-MDa plasmid in HD1-25 is probably fortuitous, and that the plasmid has no role in toxin production. The curing data in Fig. 2 also indicate that the ~130-, 51-, 44-, 9.6-, and 5.4-MDa plasmids and LDE can be lost without any obvious effect on crystal production. These results, and others

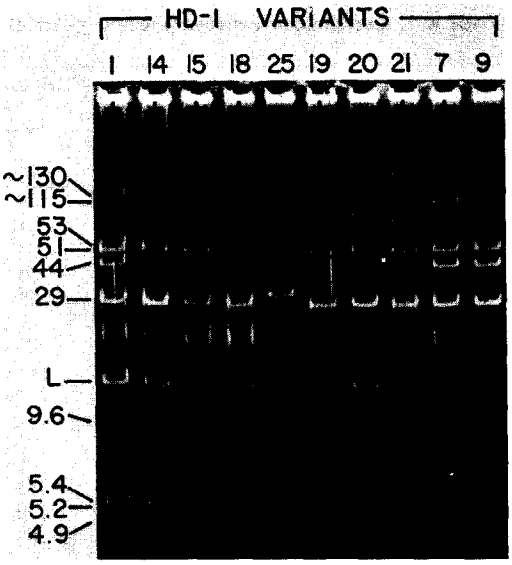

FIG. 2. Agarose gel plasmid patterns of representative mutants of strain HD-1, demonstrating the correlation of the ~115-MDa plasmid with crystalline toxin production. Molecular masses of the HD1-1 plasmids (in MDa) are listed on the left-hand margin and were obtained by using the plasmids of strain HD-2 as standards; L marks the position of the LDE. The 53- and 51-MDa plasmid doublet was earlier reported as a single 52-MDa plasmid (11). A 1.4-MDa plasmid is also present in all variants shown here, but was run off the gel during electrophoresis.

presented previously, indicate that plasmid curing, although suggesting the locations of toxin genes on plasmids, is not definite proof that a given *B. thuringiensis* plasmid carries the structural genes for delta-endotoxin (1, 11, 12), especially if there are difficulties in detecting the larger toxin plasmids (12; Carlton and González, in press). Transfer of a putative toxin plasmid from a Cry$^+$ *B. thuringiensis* donor into a Cry$^-$ *B. thuringiensis* or *B. cereus* recipient, thereby converting the recipient into a Cry$^+$ strain which produces delta-endotoxin antigenically like that of the donor strain, provides stronger evidence that the transmitted plasmid carries the toxin structural genes (1, 9–11).

The 44-MDa plasmid, although not required for toxin production, may contribute to crystal formation. Minnich and Aronson (17) recently reported that our Cry$^-$ mutant HD1-9 formed crystals when grown at 25°C, but not at 30°C. We have confirmed their observation; however, variants such as HD1-25 and HD1-20, which lack the 44-MDa plasmid and contain what is possibly a deleted form of the ~115-MDa plasmid, are Cry$^-$ at both 25 and 30°C. Variant HD1-10 (not shown), derived from HD1-9 by loss of the 44-MDa plasmid, is also Cry$^-$ at both

temperatures. Recent results indicate that HD-1 toxin production is controlled by toxin genes on both the 44- and ~115-MDa plasmids (T. Yamamoto, J. M. González, Jr., and B. C. Carlton, manuscript in preparation).

Unusual nutritional requirements of *B. thuringiensis* strains. In the course of this study, some quirks of individual *B. thuringiensis* subspecies became evident. For example, when cells were streaked on SCG agar (12) for Eckhardt slot-lysate electrophoresis, strains of certain subspecies failed to grow. Supplementation of SCG agar with additional nutrients (tryptophan, glutamine, vitamins, etc.) led to full growth of the auxotrophic strains and satisfactory plasmid visualization. Requirements for tryptophan, nicotinic acid, and thiamine were identified in various strains. All H-5ab *galleriae* strains, such as HD-8 (Fig. 1b), required nicotinic acid. The requirement of nicotinic acid by H-5ab *galleriae* strains has been reported (13, 15). The two aflagellar (*wuhanensis*) strains, HD-525 and HD-572 (Fig. 1d), also required nicotinic acid. This finding, together with their similarity in plasmid array to HD-8, suggests that *wuhanensis* probably arose from an H-5ab *galleriae* strain through loss of flagella. The two H-10 *darmstadiensis* strains, HD-146 and HD-498 (Fig. 1a), both required tryptophan. Finally, the H-17 *tohokuensis* strain, HD-866 (Fig. 1d), required thiamine for growth.

Strains of subspecies H-4ac and H-6 formed unstable spores on NSM agar. Although sporulation appeared normal, the spores began to become phase dark and to lose their viability within days. However, it was observed that spores grown on SCG agar were much more stable. Subsequently, these strains were grown on NSM agar containing glucose (0.1 to 0.2%) and were found to produce stable spores (judged by their ability to remain phase bright after lysis of the sporangia). These preliminary results indicate that some *B. thuringiensis* strains may require glucose (or perhaps any fermentable carbohydrate) for normal sporulation.

The requirement of H-10 strains for tryptophan is not surprising, since there have been many reports of the inability of *B. thuringiensis* to grow in defined media unless one or more amino acids are added (15, 18). However, this is not true for all *B. thuringiensis* strains. Preliminary experiments (results not shown) have revealed that strain HD-2 (H-1) can grow (although slowly), sporulate, and make crystals in a simple defined medium containing glucose, sodium phosphate, ammonium chloride, and small amounts of other salts. Therefore, at least HD-2 appears to have no amino acid, vitamin, or other auxotrophic requirements. On the other hand, other strains, such as HD-1 (H-3ab), were tested on the same minimal medium and grew very poorly, suggesting that true amino acid auxotrophies are present in *B. thuringiensis* strains other than those of serotype H-10. When adequate glucose-ammonium salts minimal media for *B. thuringiensis* strains are developed, the patterns of amino acid, vitamin, and other requirements, together with the complex *B. thuringiensis* plasmid arrays, may prove to be distinctive enough to play a role in the classification and identification of strains of this important industrial microorganism.

SUMMARY

A large number of strains of *B. thuringiensis*, including every known subspecies, were examined for the presence of extrachromosomal DNA by agarose gel electrophoresis. All Cry^+ strains contained at least one large plasmid of 30 MDa or more; most strains harbored complex plasmid arrays of three or more size classes. Strains of different subspecies had distinct plasmid arrays. Strains of the same subspecies usually showed similarities in their plasmid arrays, but sometimes the plasmid arrays were very different. Plasmids implicated in crystalline delta-endotoxin production by plasmid curing or plasmid transfer were invariably large (>40 MDa). An unusual mutant of strain HD-1 (subsp. H-3ab *kurstaki*) is discussed as an example of the misleading variants that sometimes arise during attempts at curing. In this Cry^+ mutant, a toxin plasmid was only apparently cured, as deduced from the occurrence of "revertants" in which the plasmid reappeared.

Some subspecies of *B. thuringiensis* exhibited characteristic nutritional requirements; for example, strains of subsp. H-10 (*darmstadiensis*) required tryptophan for growth. Some strains appeared to require the presence of glucose in the nutrient medium for production of stable spores. The complex plasmid arrays and auxotrophic requirements of individual *B. thuringiensis* strains may be useful in *B. thuringiensis* classification and identification, as well as providing convenient markers for genetic studies.

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